Transcriptional Repression of Specific Host Genes by the Mycovirus *Cryphonectria* Hypovirus 1

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The hypovirus CHV1, which infects the plant-pathogenic fungus *Cryphonectria parasitica*, causes a distinct range of symptoms in its host that include reduced virulence expression, reduced sporulation, and reduced pigment production. The virus, however, has little or no effect on fungal growth in culture. The visual symptoms are associated with reduced accumulation of a small number of host mRNAs and proteins. Four of the host genes encoding these down-regulated mRNAs have been characterized; they include two genes encoding a fungal sex pheromone (Vir1 and Vir2), a gene encoding an extracellular laccase (Lac1), and a gene encoding a cell wall hydrophobin (Crp). Expression of most other host proteins appears to be unaffected by the virus. These four genes can serve as reporter genes in studies of the effect of the virus on host gene expression. It is hypothesized that the four genes are coordinately down-regulated by the virus and probably are associated in a regulatory cascade. This hypothesis was tested by measuring the relative transcription rate of each gene in virus-infected and uninfected isogenic strains of the fungus by using nuclear run-on assays. The effects of the virus on transcription of these genes generally mirrored the observed effects of the virus on relative accumulation of the mRNAs of each gene. Although repressed transcription cannot account for all of the effects of the virus on mRNA accumulation of these four reporter genes, it is the predominant effect.

The double-stranded RNA virus *Cryphonectria* hypovirus 1 (CHV1), which infects the plant-pathogenic ascomycete *Cryphonectria parasitica*, is of interest because it perturbs host developmental processes such as sporulation and virulence and thus acts as an effective biological control agent against this important plant disease-causing fungus. CHV1-infected strains express symptoms of reduced virulence (hypovirulence), reduced asexual and sexual sporulation, and reduced pigment production. Choi and Nuss (4, 5), used infectious cDNA clones of the virus to clearly demonstrate that the viral genome is responsible for these symptoms. The mechanism by which the virus causes these symptoms is not known, but it has been shown that expression of a limited number of specific mRNAs (22) and polypeptides (23) is repressed in virus-containing strains of *C. parasitica*. The genes encoding some of these proteins have been cloned, and it has been demonstrated that the virus down-regulates expression of these genes at the mRNA level (25, 37).

Regulation of eukaryotic mRNA accumulation can occur at several levels: transcription (7, 18, 29), posttranscriptional processing (11), or mRNA turnover rate (31). A combination of these mechanisms is probably used to regulate the abundance of most mRNAs (2, 20, 27, 30). Transcription run-on has been used in plant, fungus, and animal systems as a method to unambiguously study that gene regulation occurs at the transcriptional level (12, 18, 21, 28, 32, 36). By contrast, reporter gene fusions, which are very useful in the study of gene expression, rely upon the complete transcriptional and translational apparatus of the cell for expression and thus cannot distinguish between transcriptional and other levels of mRNA regulation.

In the studies described here, we have used transcriptional run-on methods to compare transcription of four virus-regulated fungal genes in isogenic strains that differ only by the presence or absence of the virus (22). The four genes used in this study are each down-regulated by the virus at the level of mRNA accumulation in a virus-infected strain (UEP1) compared with an isogenic virulent strain (EP155/2). These genes include two putative mating-type A pheromone genes, Vir1 and Vir2 (37), which are expressed as mRNAs in EP155/2 but are undetectable in UEP1. Similarly, expression of the cryparin gene (Crp), which encodes an abundantly expressed cell surface hydrophobin (1, 38), is reduced by about 50 to 70% in UEP1 compared with EP155/2. Finally, the laccase 1 gene (Lac1), a gene encoding an extracellular polyphenol oxidase, is also down-regulated by about 50 to 70% in CHV1-infected strains of the fungus (15, 25, 26).

These four genes can be used as molecular reporters in studies of the effects of CHV1 on its host. We hypothesize that these genes are coordinately regulated and that the virus specifically perturbs their normal regulation. The best model for regulatory linkage of such diverse genes is a transcriptional cascade in response to environmental or developmental signals. We report here that transcription of each of the cloned reporter genes is significantly reduced in the virus-infected strain compared with an isogenic healthy control. The degree of transcriptional down-regulation of the four genes is consistent with the relative virus-caused reduction of mRNA accumulation of the genes.

**MATERIALS AND METHODS**

*Strains and plasmids.* *C. parasitica* EP155/2, a single-spore isolate of virulent strain EP155 (ATCC 38755), and the isogenic strain UEP1 were used in these studies (22). The fungal stocks were maintained on silica gel and stored at −20°C. The clones of genes *Vir2* (37), *Crp* (38), and *Lac1* (25) have been described previously. *Vir1* (37a) is a gene that, like *Vir2*, encodes a putative pheromone of *C. parasitica*. The genes differ in their sequence, with the exception of an identical 83-bp open reading frame found in both. The *Vir1* transcript is about 800 nucleotides (nt) long. The glyceraldehyde phosphate dehydrogenase gene (*Gpd*) was cloned from EP155/2 by PCR with the sequence reported by Choi and Nuss (3) to direct primer-initiated synthesis. The rRNA clone used is...
from *Neurospora crassa* (10). Plasmids were propagated in DH10B (Bethesda Research Laboratories, Bethesda, Md.).

**Isolation of DNA.** DNA was prepared as described by Zhang et al. (37), except that plasmid DNA was treated with 50 μg of RNase A (Sigma, St. Louis, Mo.) per ml at 37°C for 30 min followed by 100 μg of proteinase K (Sigma) per ml at 37°C for 15 min. The DNA was then extracted with phenol-chloroform and precipitated with ethanol in the presence of ammonium acetate. DNA concentra-
tions were estimated by fluorometry, and 2 μg of insert DNA per slot was transferred onto GeneScreen Plus membrane (DuPont, Boston, Mass.), as specified by the manufacturer, with a slot blot template (Bio-Rad, Hercules, Calif.) and cross-linked to the membrane by UV treatment.

**Isolation and quantitation of nuclei.** Liquid cultures of EP155/2 and UEP1 were grown and harvested as described by Zhang et al. (37). All isolation steps were performed at 4°C. Fresh mycelia (10 g) were chilled on ice and homogenized in a 360-ml chamber of a Bead Beater cell disruptor (Biosepic Products, Bartsville, Okla.) together with about 100 g of 0.5-mm-diameter glass beads and enough extraction buffer to exclude air. The buffer (a modified version of that described in reference 21) contained 100 mM KCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 4 mM spermidine, 1 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and 17% (w/v) sucrose. The hy-
phage were disrupted by three 30-s bursts with 45-s intervals. An ice-water jacket kept the extraction mixture cold during the procedure. The homogenate was decanted from the beads and filtered through four layers of Miracloth (Calbio-
chem, La Jolla, Calif.) and one layer of 20-
m-pore-size nylon mesh (Spectrum Products, Houston, Tex.). The homogenate was centrifuged at 6,000 × g for 15 min. The DNA was then extracted with phenol-chloroform and precipitated with ethanol in the presence of ammonium acetate. DNA concen-
trations were estimated by fluorometry, and 2 μg of insert DNA per slot was transferred onto GeneScreen Plus membrane (DuPont, Boston, Mass.), as specified by the manufacturer, with a slot blot template (Bio-Rad, Hercules, Calif.) and cross-linked to the membrane by UV treatment.

**Quantification of mRNA abundance.** Total RNA was isolated as previously described (22), and 3 μg per slot was blotted onto GeneScreen Plus membrane as specified by the manufacturer (DuPont). The blots were hybridized with probes generated by random priming of linearized cDNA clones of *Gpd*, *Lac1*, *Vir1*, *Vir2*, and *Crp*, and *rRNA*. The relative hybridization of the clones to the total RNA content on each slot was quantified by scanning the exposed X-ray film with an LKB Ultrascan XL densitometer. Only signals determined to be in the linear response range were used for data analysis.

**RESULTS**

**Relative accumulation of reporter gene mRNA.** Total RNA was isolated from strains UEP1 and EP155/2 between 1.5 and 6 days after inoculation and analyzed for mRNA accumulation by using Northern (RNA) blots. These blots were probed separately with linearized cDNA clones of the genes encoding *Crp*, *Lac1*, *Vir1*, *Vir2*, *Gpd*, and *rRNA* (Fig. 1). *Gpd* and *rRNA* were used as controls, since the expression of these genes was not affected by CHV1. Results showed that the mRNA of *Vir1* and *Vir2* could not be detected in the virus-containing strain UEP1 at any of the times tested, although both genes were highly expressed in the uninfected strain. Likewise, *Lac1* mRNA was barely detectable in the virus-containing strain but was abundantly expressed during the active growth phase of the uninfected strain, as previously reported (25). *Crp* mRNA accumulation was reduced in UEP1 compared with EP155/2, particularly after 3 days of growth.

**Optimization of the transcription reaction.** To identify times during the growth cycle of the fungus that were most favorable for transcription analysis, we tested nuclei for transcription activity at various times after inoculation. Nuclei isolated 2 days after inoculation incorporated labeled nucleotides more rapidly in the nuclear run-on assay than did those isolated 1.5, 3, or 4 days after inoculation (data not shown). Because viral effects on mRNA accumulation were also detectable by 2 days, this time was chosen for the studies reported.

The nuclear run-on reaction was optimized for incubation time, salt concentration, and the effect of heparin. The optimal cation concentration was determined by varying the (NH₄)₂SO₄ and KCl concentrations from 0 to 200 mM and was found to be 100 mM, consisting of 50 mM (NH₄)₂SO₄ and 50 mM KCl (data not shown). This salt optimum is similar to that reported for polymerase II (polII) of *Neurospora crassa* (35). Addition of heparin to the reaction mixture increased incorporation over the time course (Fig. 2). At 30°C, nucleotide incorporation increased for the first 10 to 15 min and then declined.

**Inhibition of transcription by α-amanitin.** Inhibition of the nuclear run-on reaction by α-amanitin was performed with nuclei isolated from EP155/2 2 days after inoculation. The concentration of α-amanitin was varied from 0 to 700 μg/ml with all other reaction conditions remaining constant. The results showed that the incorporation of labelled nucleotide was sensitive to the α-amanitin (Fig. 3); however, 500 μg/ml was necessary to reduce incorporation to background levels. Reactions performed in the presence of 30 and 50 μg of α-amanitin per ml still generated transcripts which hybridized to the reporter genes, *Gpd*, and *rRNA* (data not shown). When products of reaction mixtures containing 500 μg of α-amanitin
per ml were used to probe blotted DNA, rRNA expression was apparently unaffected but expression of the reporter genes, presumably transcribed by RNA polII, was significantly reduced (data not shown). Mammalian polIII is sensitive to as little as 2 μg of α-amanitin per ml (36), but N. crassa (34, 35) polIII is relatively insensitive to α-amanitin, being inhibited only 80% by 1 mg of α-amanitin per ml. Our results showed that polIII of C. parasitica was also relatively insensitive to inhibition by α-amanitin compared with that of most eukaryotes, but our results do support the expectation that Vir1, Vir2, Crp, Lac1, and Gpd are products of polII-directed transcription.

Relative transcription rates of genes of the virulent strain, EP155/2. Products of run-on experiments performed with nuclei isolated from 2-day-old cultures of EP155/2 were used to probe DNA blots of the reporter genes. The resulting data are reported as the ratio of the signal for transcripts from the reporter genes to that of transcripts from Gpd (Fig. 4A). The transcription rates of each of the genes of EP155/2 corresponded generally to the relative abundance of the respective mRNAs as detected by Northern blot analysis (Fig. 1 and 4A). Crp was the most abundant polII transcript of those examined, and it is, in our experience, also the most abundant mRNA among the reporter genes 2 days after inoculation (38).

Down-regulation of transcription in strain UEP1. Data collected from a run-on experiment with nuclei isolated from the virus-infected strain (UEP1) are reported in Fig. 4B. These results were similar to those reported in Fig. 4A with respect to the hierarchy of expression of the four reporter genes. There was, however, considerably less transcription of Vir1, Vir2, and Crp relative to that of the same genes of EP155/2. Transcription of Lac1 in UEP1 was also reduced compared with that in EP155/2 but not as dramatically as that of the other reporter genes.

A quantitative comparison of the down-regulation of the reporter genes in the CHV1-infected strain UEP1 with that of the genes in the normal strain, EP155/2, is provided in Fig. 5A. There was a significant down-regulation at the transcriptional level of all four reporter genes in the CHV1-infected strain. The validity of using Gpd to normalize the data was demonstrated by including rRNA in the comparisons: the results were the same whether normalized to Gpd or rRNA transcripts, and the expression of both transcripts was unaffected by CHV1. The reporter genes differed in their relative degree of transcriptional down-regulation. Transcription of Vir1 and Vir2 in the virus-infected strain was reduced to 25 to 35% of that in the normal strain. This contrasts with our consistent inability to detect mRNAs of these genes in UEP1 (Fig. 1). The transcription of Crp and Lac1 was not as greatly affected by the virus as that of Vir1 and Vir2; transcript levels of these genes in UEP1 were about half those in EP155/2 (Fig. 5A).
reporter genes fell into two groups based on the effects of CHV1 on transcription and mRNA accumulation. In the first group, the mRNAs of Vir1 and Vir2 are generally not detectable in UEP1 (37), while their transcription rate is reduced to 70% compared with that of EP155/2; genes in the second group, comprising Crp and Lac1, have mRNA levels in UEP1 that are usually about 30% of those in EP155/2 (25, 38), while their transcription rate in UEP1 is reduced about 50% compared with that of EP155/2.

An aliquot of the mycelium harvested from 2-day-old cultures to prepare nuclei for the run-on experiments was used to extract total RNA to determine in parallel the relative accumulation of mRNAs corresponding to the reporter genes (Fig. 5B). In this experiment, rRNA transcription was used to normalize the results between the two strains and experiments. The results with either Gpd or rRNA as the standard were essentially interchangeable and are consistent with those of the experiment in Fig. 5A. The transcription of Crp was significantly inhibited in UEP1 by CHV1 at 2 days after inoculation, but the Crp mRNA accumulation at this time was similar in UEP1 and EP155/2. Thus, although Lac1 and Crp transcriptions were both down-regulated by about the same amount, this similarity in transcriptional inhibition by the virus was not
yet reflected in the amount of mRNA that had accumulated 2 days after inoculation. Viral effects on transcription of Lac1 must temporally precede the effects on Crp, affecting mRNA accumulation of Lac1 sooner than that of Crp (Fig. 5).

**DISCUSSION**

There are a variety of models to explain how trans-acting elements encoded by viruses interact with their hosts to perturb transcriptional regulation. The best-known examples are v-fos and v-jun, viral oncogenes whose cellular equivalents are c-fos and c-jun, respectively. These genes encode proteins which form the nuclear transcription factor, AP-1 (13). The AP-1 complex is involved in activating a specific class of genes, some of which are involved in cell growth and differentiation. Fibroblast cells which are virally transformed show disrupted expression of the normal protein patterns and differentiation processes of the cell. The exact mechanisms by which this occurs are unknown (14). Poliovirus, a member of the Picornaviridae, is known to specifically inhibit host cell RNA synthesis by inactivating RNA polII, polIII, and polIII. Poliovirus also inactivates translation of mRNA into proteins by cleaving the cap-binding protein complex of the host cell (8). Recent work has shown that polIII activity of poliovirus-infected HeLa cells is perturbed by a virus-encoded protease, 3C\(^{\text{pro}}\), which cleaves the TATA-binding protein, a component of a human transcription factor (TFIID) (6). This type of down-regulation of transcription by a virus is not directly comparable to what we observe in CHV1-infected C. parasitica, since the former is a much more global effect than the specific down-regulation of a limited number of host genes caused by CHV1. The effect of CHV1 on its host is more reminiscent of that of the adenovirus E1A gene down-regulation of a specific group of its host’s genes, the major histocompatibility complex class I genes, by stimulating a negative regulatory element (24). The adenovirus protein E1A has also been shown to drive cells into DNA synthesis and cell cycle progression by interaction with specific cell cyclin proteins. Many of the DNA tumor viruses, such as adenovirus, simian virus 40, human papillomavirus, and polyomaviruses, do this by inactivating cell growth suppressors (9).

Infection of C. parasitica by CHV1 results in a number of easily detected phenotypic changes such as reduced sporulation, pigmentation, and virulence, but growth in culture remains normal. These phenotypic changes, while facilitating experimental approaches to the study of this virus, are subjective and hence are inadequate for use in molecular studies of how CHV1 affects C. parasitica. At the molecular level, the virus is associated with a reduced accumulation of a limited number of detectable fungal mRNAs and proteins, while expression of most host proteins is unaffected by the virus (22, 23). To investigate the mechanism of virus-induced symptom expression, four of the down-regulated genes were cloned and their functions were investigated. These four genes can now be used as molecular reporters to study how the virus is able to reduce the accumulation of their mRNAs in infected cells. The simplicity of the host, the ability to construct infectious cDNA clones of the virus (5), and the availability of a number of molecular reporters of virus infection make this a useful system to study the effects of a virus on its eukaryotic host.

The discovery that Vir1 and Vir2 are sex pheromones (37a) identifies the well-characterized fungal mating pathway as being perturbed by the virus. In the ascomycetes, the immediate regulator of sex pheromones is the mating-type (Mat) locus, which acts as a positive regulator of pheromone expression (16). Crp, another of the reporter genes, encodes a cell surface hydrophobin, probably a cell wall protein that plays a role in the structure or organization of cell surfaces. Although the protein accumulates primarily in sporulation structures in the stroma of infected trees (1), its regulation is not likely to be under the control of the Mat locus. Likewise, Lac1, while possibly coordinately regulated with Crp, is unlikely to be regulated by Mat. The experiments reported here were initiated to address the question whether regulation of the reporter genes is consistent with the hypothesis that the virus regulates each by a common mechanism, with that mechanism being perturbation of a normal regulatory cascade. If this is the case, a genetic approach can be used to identify common regulatory genes. The first step in testing this hypothesis was to determine if the reduced accumulation of each of these genes in virus-infected strains was a result of reduced transcription.

It is clear from our results that transcription of each of the reporter genes was repressed in the virus-containing strain relative to that in the isogenic uninfected strain. Transcriptional down-regulation of Vir1 and Vir2 by the virus was more marked than that of Lac1 and Crp, just as mRNA accumulation of Vir1 and Vir2 was more strongly affected by the virus than was mRNA accumulation of Lac1 and Crp. The transcriptional down-regulation of the reporter genes was not sufficient, however, to account for all of the reduction in mRNA accumulation caused by the virus. For instance, Vir1 and Vir2 mRNA levels are usually below the level of detection in UEP1, yet measurable transcription of these genes was detected. Transcription of Crp, on the other hand, was much lower than that expected from the mRNA accumulation data in Fig. 5, but this is likely to be the result of a temporal effect of viral down-regulation of this gene. It has been our observation (Fig. 1) (38) that effects of CHV1 on expression of Crp vary with growth stage in culture. The results of our transcription study, relative to mRNA accumulation levels, may represent a lag between detection of the transcriptional down-regulation and the reduced accumulation of its mRNA. Although it is not unreasonable to assume that posttranscriptional regulation may also be involved in the regulation of these genes (2), our data clearly demonstrate that the most significant factor affecting mRNA accumulation of these genes is transcriptional down-regulation.

Studies of the effect of the virus on Lac1 expression have suggested that this gene is regulated by both stimulatory and inhibitory pathways (17) and that the virus inhibits the stimulatory pathway. The use of various inhibitors of inositol triphosphate and calcium-dependent signal pathways led Larson et al. (17) to speculate that CHV1 affects expression of Lac1 by disruption of signal transduction. The studies of Larson et al. (17) have shown the value of using a reporter gene to investigate the mechanisms of viral perturbation of host gene function. Since in their studies only a single reporter gene was used, it is not clear whether their results can be extrapolated beyond Lac1.

We have shown that transcription of a limited number of host genes is repressed in CHV1-infected strains of the fungus. The four genes we have selected as molecular reporters to study the effect of CHV1 on its host are similar in that they are not constitutive and at least three of them are associated with sporulation of the fungus. They are each different, however, in the timing of their expression relative to the fungal growth cycle in liquid culture (Fig. 1). It is our hypothesis that these genes are normally coordinately regulated at some stage in the development of the fungus and that viral perturbation of this regulation leads to their repression, resulting in viral symptom expression.
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REFERENCES


